

SUMMARY OF RESEARCH

PHYSIOLOGICAL MATURATION OF REGENERATING HAIR CELLS

NRA: NAG2-1343 (Research Opportunities in Space Life Sciences)

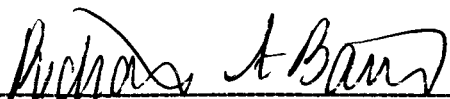
PRINCIPAL INVESTIGATOR: Richard A. Baird, Ph.D.

INSTITUTION: Harold W. Siebens Hearing Research Center
Central Institute for the Deaf
4560 Clayton Avenue
St. Louis, MO 63110-1549

TELEPHONE: (314) 862-3511
FAX: (314) 977-0030
EMAIL: rbaird2002@earthlink.net

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APPROVED:



Richard A. Baird, Ph.D., Director of Research
Harold W. Siebens Hearing Research Center
Central Institute for the Deaf

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Date

Summary of research

(1) Overview

The bullfrog sacculle, a sensor of gravity and substrate-borne vibration, is a model system for hair cell transduction. Saccular hair cells also increase in number throughout adult life and rapidly recover after hair cell damage, making this organ an ideal system for studying hair cell development, repair, and regeneration. We have used of hair cell and supporting cell immunocytochemical markers to identify damaged hair cells and hair cell precursors in organotypic cultures of the bullfrog sacculle. We then used an innovative combination of confocal, electron, and time-lapse microscopy to study the fate of damaged hair cells and the origin of new hair cells after gentamicin ototoxicity in normal and mitotically blocked saccular cultures. These studies have shown that gentamicin ototoxicity produces both lethal and sublethal hair cell damage. They have also shown that hair cell recovery in this organ takes place by both the repair of sublethally damaged hair cells and by the replacement of lost hair cells by mitotic regeneration.

In parallel studies, we have used biophysical and molecular biological techniques to study the differentiation and innervation of developing, repairing, and regenerating hair cells. More specifically, we have used RT-PCR to obtain the bullfrog homologues of L-type voltage-gated calcium (L-VGCC) and large-conductance Ca^{2+} -activated potassium (BK) channel genes. We have then obtained probes for these genes and, using *in situ* hybridization, begun to examine their expression in the bullfrog sacculle and amphibian papilla. We have also used fluorescent-labeled channel toxins and channel toxin derivatives to determine the time of appearance of L-type voltage-gated calcium (L-VGCC) and Ca^{2+} -activated potassium (BK) channels and to study dynamic changes in the number, distribution, and co-localization of these proteins in developing, repairing, and regenerating hair cells. Using time-lapse microscopy, we are also studying the dynamic relationship between ion channel clustering and synaptic formation in hair cells and afferent neurons.

In future studies, we will determine when hair cell precursors acquire electrical tuning, and, using whole-cell patch-clamp techniques, identify and characterize their L-VGCC and BK currents. We will also use biophysical techniques to determine the number of L-VGCC and BK channels and the size and gating kinetics of their underlying L-VGCC and BK conductances, correlating these variables with the amplitude and frequency of membrane oscillations produced by intracellular current steps. We expect these studies to determine how hair cells regulate ion channel expression to achieve specific physiological responses.

(2) Molecular biological studies of calcium and potassium channel genes

Electrical tuning in many hair cells is determined by the interplay of L-VGCC and BK channels. To study the acquisition of these channels in the bullfrog inner ear, we designed degenerate primers against conserved sequences of their α -subunit genes and identified, using RT-PCR, their bullfrog homologues.

L-VGCC channels – PCR products derived from primer amplification of bullfrog brain and inner ear cDNA were cloned, examined with restriction digestion, and sequenced to verify their identity. Amplification of the insert sequences produced a single 600-bp band. There was a 70% homology between the nucleotide sequence of this fragment and a region between the first and second repeats of the chick α_{1D} subunit. Alignment with the chick α_{1C} subunit had less homology, with only 45 identical nucleotides in a 70-nucleotide aligned sequence. With three exceptions, every nucleotide in the bullfrog sequence was found in the chick sequence; the chick sequence also contained two spliced-in sequences lacking in the bullfrog sequence.

BK channels – Degenerate primers against a splice site in the C-terminus immediately downstream of the last transmembrane domain were designed by aligning nucleotide sequences for *tslo*, *cslo*, *mslo*, and *hslo* genes. Amplification of these primers produced bands of 207- and 231-bp, depending on the absence or presence of an alternately spliced sequence implicated in frequency coding in the chicken cochlea. These bands were identical to the *insertless* and *SRKR* inserts seen in chick brain and inner ear. There was an 85% homology

between the nucleotide sequences of these products with a region in the C-terminus of *cslo*, suggesting that these clones encoded a bullfrog BK homologue.

High-stringency screening of a bullfrog saccular cDNA library obtained from Drs. Stefan Heller and Jim Hudspeth at The Rockefeller University with DIG-labeled probes at 50°C confirmed that both of the above channel genes are expressed in the bullfrog sacculus. We have sequenced clones from this library screening to obtain the minimal coding sequence and to identify the splice variants of these channel genes. We are now preparing DIG-labeled probes against these ion channel genes and, using *in situ* hybridization, study the expression of these genes in developing hair cells, repairing hair cells, and regenerating hair cells.

(3) Fluorescent studies of calcium and potassium channels

In collaboration with Dr. Lane Brown of Oregon Health Sciences University, we have fluorescently labeled apamin and a mutated form of charybdotoxin (CTX-R19C) obtained from Dr. Chris Miller of Brandeis University. We then verified with whole-cell patch clamp recordings that these labeled toxins retained their specificity for large-conductance (BK) and small-conductance (SK) Ca^{2+} -activated potassium channels, respectively. To study how hair cells regulate these channels during development, we used these toxin derivatives and fluorescently-labeled dihydropyridines (FL-DHP) and ω -conotoxin GVIA (FL-GVIA), specific ligands of L-type (L-VGCC) and N-type (N-VGCC) voltage-gated calcium channels, to determine the time of appearance, distribution, and co-localization of calcium and potassium channels in mature and immature hair cells. These studies have been carried out in the bullfrog sacculus and in the amphibian papilla, a tonotopically organized detector of low-frequency sound. Both the sacculus and the amphibian papilla have restricted terminal fields and exhibit hair cell repair and regeneration after trauma-induced damage, making them excellent systems for studying synaptogenesis and re-innervation.

Our results indicate that clusters of L-VGCCs (1) appear before BK channels, (2) increase in size and migrate to more basal regions, and (3) co-localize in a fixed ratio with BK channels during development. Apamin-labeled clusters, although seen in mature hair cells, were seldom seen in immature hair cells. They were also not co-localized with FL-DHP labeled clusters. Supporting cells in the central sacculus did not display labeling to any channel marker. N-VGCCs, although seen on afferent and efferent neurons, were not observed on mature hair cells or supporting cells. We are now making similar observations on repairing and regenerating hair cells in the bullfrog sacculus and amphibian papilla to determine how these cells acquire and organize their complement of L-VGCC and BK channels. Using multi-photon microscopy, we have also begun to study *dynamic* changes in ion channel clustering in mature and immature hair cells.

Using immunocytochemical markers, we have also examined the relationship between these ion channels and the presynaptic active zone delineated by the neuronal terminal. Clusters of L-VGCC and BK channels, composed of varying numbers of co-localized ion channel puncta, were closely associated with presynaptic SNARE proteins and neuronal terminals, often forming a ring about the presynaptic active zone. The number of these co-localized clusters was correlated with the size of the presynaptic active zone, implying that hair cells vary their number of BK channels by varying both the number of synapses and the number of ion channel clusters per synaptic zone. We have also begun to study dynamic changes in ion channel clustering and synaptic formation in mature and immature hair cells. We will then examine trauma-induced changes in channel clustering and synaptic remodeling in repairing and regenerating hair cells.

(4) Immunocytochemical studies of ion channels

We have used pan-alpha and subunit-specific antisera (Alamone Laboratories) to verify the distribution and co-localization of L-VGCC and N-VGCC channels and small-conductance Ca^{2+} -activated (SK) potassium channel on hair cells and neuronal terminals in normal saccular cultures. In collaboration with Dr. David McCobb of Cornell University, we have also used pan-

alpha and splice-variant antisera to confirm the distribution of large-conductance Ca^{2+} -activated potassium (BK) channels on saccular hair cells. We have also determined that FL-DHP and FL-CTX immunolabeling in saccular hair cells is co-localized with several vesicular and membrane-bound presynaptic receptor proteins, including synaptotagmin, SNAP-25, syntaxin, and synapsin.

(5) Significance of experimental results

We have created and well-defined organotypic culture systems to study in isolation mitotic and non-mitotic events in hair cell recovery. More recently, this approach has also been used by other investigators to study mitotic and non-mitotic mechanisms of hair cell recovery in the mammalian and chick inner ear organs. We are also using this approach to examine mitotic and non-mitotic recovery in the bullfrog auditory organs in isolation in our studies of afferent synaptogenesis. We have used morphological and immunocytochemical criteria to identify lethally and sublethally damaged hair cells and distinguish developing, repairing, and regenerating hair cells. This was a major problem with previous studies of hair cell recovery because these cell populations have similar morphology and could not be distinguished solely by morphological criteria. After several 'false-starts', which revealed the advantages and disadvantages of several approaches, we ultimately developed a fluorescent time-lapse system that has the necessary sensitivity, resolution, and long-term stability to address the questions posed by our experiments. By documenting the *dynamic* morphological and immunocytochemical changes that occur in hair cells and supporting cells after hair cell damage, our studies have begun to rigorously define the boundary between hair cell damage, repair, and programmed cell death and to shed light on the cellular and molecular mechanisms that regulate gene expression and protein synthesis in damaged hair cells.

Unfortunately, our understanding of how developing, repairing, and regenerating hair cells differentiate and are (re-)connected to the CNS is incomplete. This is largely because it has been difficult to *identify* nascent synapses and to *dynamically* follow changes in synaptic organization. We are therefore only beginning to understand how conventional synapses are induced, stabilized, and maintained during normal development. Although the functional recovery of central auditory and vestibular reflexes after ototoxicity or sound exposure implies that new HCs are (re-)innervated by afferent neurons, we have also only begun to identify the molecular mechanisms that regulate synaptogenesis at these specialized ribbon synapses during regeneration and injury-induced synaptic remodeling. A clear understanding of these processes is necessary before we can determine when, where, and how biological strategies for stimulating the (re-)innervation of developing, repairing, or regenerating HCs would be most effective. Biological strategies for stimulating neuronal outgrowth, directing neurons to new HC targets, and promoting post-embryonic synaptogenesis would also significantly improve the effectiveness of cochlear implants.

(6) Subject Inventions

None.

(7) Publications

Invited conference presentations

- Auditory Neuroscience Colloquium: Development, Transduction, and Integration (National Academy of Sciences), Irvine, CA, May, 2000.
- The Vestibular Labyrinth in Health and Disease (New York Academy of Sciences), St. Louis, MO, November, 2000.
- New Frontiers in the Amelioration of Hearing (A tribute to Jim Miller), St. Louis, MO,

March, 2001.

- Symposium on Hair Cell Rescue, Repair, and Regeneration Symposium, American Academy of Audiology annual meeting, San Diego, CA, April, 2001.
- EURESCO conference on regeneration, Castelvecchio pascoli, Italy, September, 2002.

Manuscripts (published)

Baird, R.A., Steyger, P.S., and Schuff, N.R. (1997) Intracellular distributions and putative functions of calcium-binding proteins in the bullfrog vestibular otolith organs. *Hear. Res.* 103: 85-100.

Steyger, P.S., Burton, M.D., Hawkins, J.R., Schuff, N.R., and **Baird, R.A.** (1997) Calcium-binding proteins are early markers of non-mitotically regenerating vestibular otolith hair cells. *Int J. Develop. Neurosci.* 15, No. 4/5: 417-432.

Steyger, P.S., Gillespie, P.G., and **Baird, R.A.** (1998) Myosin I β is located at tip-link anchors in adapting and non-adapting vestibular hair bundles. *J. Neuroscience* 18: 4603-4615.

Baird, R.A., Burton, M.D., Lysakowski, A., Fashena, D.S., and Naeger, R.A. (2000). Hair cell repair and regeneration in mitotically blocked saccular cultures. *PNAS* 97: 11722-11729.

Baird, R.A., Burton, M.D., and Johnson, S.N. (2003) Hair cell recovery in normal and mitotically blocked cultures of the bullfrog saccule. *Seminars in Hearing* 24: 165.

Manuscripts (in preparation)

Baird, R.A., Burton, M.D., Johnson, S.N., and Hooper, R.A. (2003) Ongoing and gentamicin-induced cell proliferation in the bullfrog saccule. *J. Comp. Neurol.* (*In preparation*).

Baird, R.A., Burton, M.D., Lysakowski, A., Simmons, D.D., Johnson, S.N., and Hooper, R.A. (2003) Hair cell recovery in mitotically blocked cultures of the bullfrog saccule. *JARO* (*In preparation*).

Baird, R.A., Burton, M.D., Hennig, A.K, Johnson, S.N., and Hooper, R.A. (2003) Brn-3c expression in developing, repairing, and regenerating hair cells in the bullfrog saccule. *J. Comp. Neurol.* (*In preparation*).

Simmons, D.D., **Baird, R.A.**, Burton M.D., Johnson, S.N., and Hooper, R.A. (2003) Hair cell recovery in the sound-damaged bullfrog amphibian papilla monitored by otoacoustic emissions. *JARO* (*In preparation*).

Baird R.A., Johnson, S.N., Burton, M.D., and Hooper, R.A. (2003) Afferent innervation of mature and immature hair cells in the bullfrog amphibian papilla. *J. Comp. Neurol.* (*In preparation*).

Baird, R.A., Brown R.L., Simmons, D.D., Burton, M.D., Hooper, R.A., and Johnson, S.N. (2003) Distribution and co-localization of L-VGCC and BK channels in the amphibian papilla visualized with fluorescent toxin derivatives. *J. Neurosci.* (*In preparation*).

Baird R.A., Brown, R.L., Burton, M.D., Hooper, R.A. and Johnson, S.N. (2003) Ion channel clustering and synaptogenesis in developing hair cells of the bullfrog saccule. *J. Neurophysiol.* (*In preparation*)

Baird, R.A., Burton, M.D., Johnson, S.N., and Hooper, R.A. (2003) Ongoing and sound-induced cell proliferation in the bullfrog amphibian papilla. *J. Comp. Neurol.* (*In preparation*).

Abstracts

Steyger, P.S., Gillespie, P.G., and **Baird, R.A.** (1997) Myosin I β is located at tip-link anchors in adapting and non-adapting vestibular hair bundles. *Assoc. Res. Otolaryngol.* 20: 118.

Burton, M.D., Cyr, J.L., Hasson, T., and **Baird, R.A.** (2000) Cytokeratin and myosin VI immunolabeling in mitotically-blocked saccular cultures. *Assoc. Res. Otolaryngol.* 23: 241.

Elam, L.Y., Hennig, A.K. and **Baird, R.A.** (2000) Preliminary identification of L-type Ca²⁺ and BK channel genes in the bullfrog saccule. *Assoc. Res. Otolaryngol.* 23: 166.

Lysakowski, A., Burton, M.D., Price, S., and **Baird, R.A.** (2000) Ultrastructural studies of mitotically blocked saccular cultures. *Assoc. Res. Otolaryngol.* 23: 242.

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Fashena, D.S. and **Baird, R.A.** (2001) Apical endocytosis in hair cells of the bullfrog sacculle. *Assoc. Res. Otolaryngol.* 24: 243.

Burton, M.D., Brown, R.L., Johnson, S., and **Baird, R.A.** (2001) Distribution and co-localization of calcium and calcium-activated potassium channels in the bullfrog sacculle. *Molecular Biology of Hearing and Deafness Abstr.*

Baird, R.A., Burton, MD. D., and Naeger, R.A. and Johnson, S.N. (2002) Hair cell recovery in mitotically blocked saccular cultures." *Cellular and Molecular Basis of Regeneration (EURESCO conference)*, Castelvecchio – Il Ciocco, Italy.

Baird, R.A., Brown, L.A., Burton, M.D., Hooper, R.A., and Johnson, S.N. (2002) Formation of ion channel clusters and synaptic connections in amphibian hair cells. *Soc. Neurosci. Abstr.* 32.

Baird, R.A., Brown, L.A., Burton, M.D., Hooper, R.A., and Johnson, S.N. (2002) Development of ion channel clusters and synaptic connections in the bullfrog sacculle. *Barany Society XXII Meeting*, Orcas Island and Seattle, WA.

Baird, R.A., Burton, M.D., Brown, R.L., Simmons, D.D., Hooper, R.A. and Johnson, S.N. (2003) Ion channel clustering and synapse formation in the normal and sound-damaged bullfrog AP. *Assoc. Res. Otolaryngol.* 26: 13.

Burton, M.D., Brown, R.L., Simmons, D.D., Johnson, S.N., Hooper, R.A., and **Baird, R.A.** (2003) Formation of ion channel clusters and synaptic connections in the developing bullfrog sacculle. *Assoc. Res. Otolaryngol.* 26: 148.

Johnson, S.N., Burton MD, Hooper, R.A., and **Baird, R.A.** (2003) Afferent and efferent innervation in the normal and sound-damaged bullfrog AP. *Assoc. Res. Otolaryngol.* 26: 166